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Silent Mischief: Bacteriophage Mu Insertions Contaminate Products of *Escherichia coli* Random Mutagenesis Performed Using Suicidal Transposon Delivery Plasmids Mobilized by Broad-Host-Range RP4 Conjugative Machinery▽

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Random transposon mutagenesis is the strategy of choice for associating a phenotype with its unknown genetic determinants. It is generally performed by mobilization of a conditionally replicating vector delivering transposons to recipient cells using broad-host-range RP4 conjugative machinery carried by the donor strain. In the present study, we demonstrate that bacteriophage Mu, which was deliberately introduced during the original construction of the widely used donor strains SM10 λ*pir* and S17-1 λ*pir*, is silently transferred to *Escherichia coli* recipient cells at high frequency, both by *hfr* and by release of Mu particles by the donor strain. Our findings suggest that bacteriophage Mu could have contaminated many random-mutagenesis experiments performed on Mu-sensitive species with these popular donor strains, leading to potential misinterpretation of the transposon mutant phenotype and therefore perturbing analysis of mutant screens. To circumvent this problem, we precisely mapped Mu insertions in SM10 λ*pir* and S17-1 λ*pir* and constructed a new Mu-free donor strain, MFD*pir*, harboring stable *hfr*-deficient RP4 conjugative functions and sustaining replication of Π-dependent suicide vectors. This strain can therefore be used with most of the available transposon-delivering plasmids and should enable more efficient and easy-to-analyze mutant hunts in *E. coli* and other Mu-sensitive RP4 host bacteria.

Functional study of the genes of a given organism is an essential step toward biological characterization. This is generally performed by gene alteration using procedures designed to either modify or delete specific target genes or to introduce random mutations in the genome of the studied bacterium (8, 20, 24, 30). Many of these strategies rely on the integration of an exogenous fragment of DNA, such as an antibiotic marker or a transposon, taking advantage of processes involved in natural horizontal gene transfer, such as transformation, phage transduction, or bacterial conjugation (37).

One of the most popular methodologies for performing unbiased genetic screens is the analysis of the phenotypic consequences of random insertion of transposon elements. While transposon mutagenesis can now be achieved using partial or total *in vitro* procedures, the method of choice usually involves efficient conjugative transfer from a donor strain to the target recipient strain of a conditionally replicative suicide vector carrying transposon delivery machinery (19–21). Once in the recipient cell, the vector cannot replicate, allowing subsequent selection of a random transposon insertion in the bacterial genome.

Conditionally replicating plasmids of the R6K family (IncX) are very often used to deliver gene-inactivating elements in enteric bacteria (11, 28, 38). The replication of these plasmids requires the *pir*-encoded Π protein, which is usually provided in *trans* in the donor strain. In the absence of Π in the recipient strain, these plasmids cannot replicate. Therefore, these "suicide" vectors are rapidly lost but allow the integration of passenger exogenous DNA carrying selection markers through transposition or homologous recombination in the chromosomes of recipient strains. Although the delivery of these conditional replicative plasmids can be achieved through transformation, this is a highly inefficient procedure, even in transformable bacteria. Common derivatives of these plasmids that carry the *oriT* transfer origin of the broad host-range conjugative plasmid RP4 (IncPα) were therefore constructed (45). Such plasmids can be mobilized and transferred between Gram-negative and even some Gram-positive bacteria, provided that the RP4 delivery machinery is expressed in *trans* from a plasmid or from chromosomal inserted genes (17, 40).

In the early 1980s, Pühler and collaborators designed two specialized *Escherichia coli* donor strains, SM-10 and S17-1, which enable mobilization of *oriT*-containing plasmids to a broad range of recipient strains through RP4-dependent conjugative transfer (Fig. 1) (40). Later, λ*pir* prophage was introduced into both strains to enable replication of Π-dependent suicide vectors (14, 28). These two strains carry, at different

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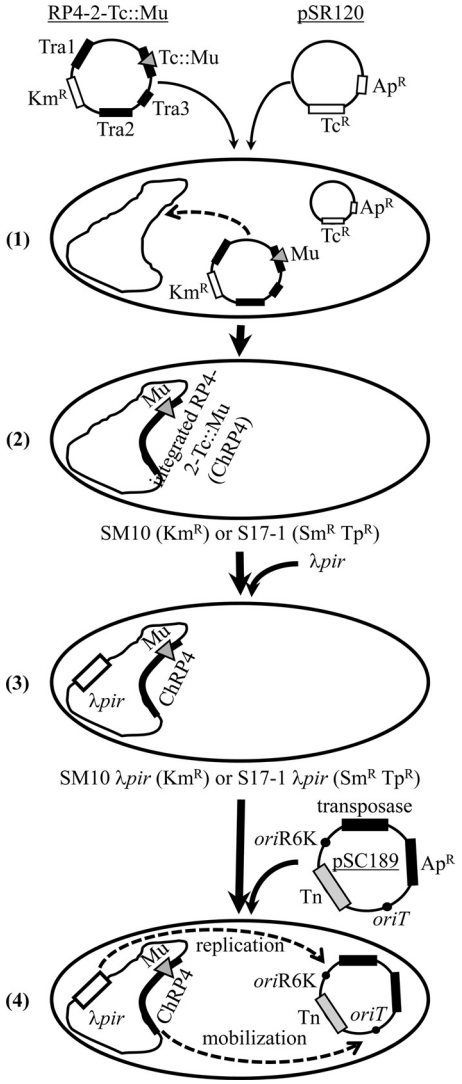

FIG. 1. History of construction of S17-1 λ*pir*(pSC189) and SM10 λ*pir*(pSC189) donor strains for transposon-based random mutagenesis using the R6K suicide plasmid pSC189. The RP4-2-Tc::Mu plasmid carries loci coding for RP4 conjugation machinery (Tra1, Tra2, and Tra3), a tetracycline resistance marker interrupted by Mu (Tc::Mu), and genetic elements coding for kanamycin resistance (Km^r). RP4-2-Tc::Mu integration into the chromosome of *E. coli* was isolated after cotransformation with the incompatible mini-RP4 plasmid pSR120 and selection for clones displaying both kanamycin and tetracycline resistance (step 1). The clones obtained were cured from pSR120, leading to SM10 (parental strain, S49-20 Km^r) or S17-1 (parental strain, *E. coli* 294 Sm^r Tp^r, following inactivation of the RP4-associated kanamycin resistance gene by Tn*7* insertion), which contain the integrated RP4-2-Tc::Mu plasmid (ChRP4), as described previously (step 2) (40). For simplicity, only a single depiction is presented for strains SM10 and S17-1. Finally, the Π-encoding gene *pir* was introduced at the λ site (step 3), and the strains were transformed with the suicide vector pSC189, which carries a *mariner*-based transposon (Tn) and its C9 transposase (transposase) and harbors the RP4-dependent origin of conjugation, *oriT*, for mobilization and the Π-dependent origin of replication, *ori*R6K (step 4) (11, 14, 28).

chromosomal locations, an RP4 derivative, RP4-2-Tc::Mu, in which the RP4 tetracycline resistance gene is interrupted by a fully functional Mu temperate prophage that could potentially undergo lytic development and liberate phage particles. These genetic procedures led to the creation of SM10 λ*pir*, which is kanamycin resistant, and S17-1 λ*pir*, which is kanamycin sensitive and streptomycin/trimethoprim resistant (Fig. 1). These strains have since been widely used for genetic analyses performed by random mutagenesis or allelic exchange in *E. coli* (4, 23, 29, 38) and many other bacteria: *Salmonella enterica* serovar Typhimurium (25, 27), *Sinorhizobium meliloti* (33), *Pseudomonas fluorescens* (52), *Erwinia carotovora* (32), *Vibrio cholerae* (28), and many more.

Although generally disregarded, it has been reported that the *oriT* located within the RP4-2-Tc::Mu region can promote *hfr* conjugative transfer of chromosomal genes, including transposable elements native to the *E. coli* donor strain (3, 40, 41). Whereas mutations of the *oriT nic* region of RP4-2-Tc::Mu prevent the mobilization of the integrated RP4 (3), there were also reports mentioning that phenotypes displayed by transconjugants obtained using S17-1 λ*pir* or SM10 λ*pir* were either unstable or not linked to the insertion event, suggesting that secondary mutational events could also have occurred (references 3, 26, and 38 and our own observations).

Here, we show that bacteriophage Mu originating from the RP4-2-Tc::Mu derivative present in S17-1 λ*pir* and SM10 λ*pir* chromosomes can transfer to *E. coli* recipient strains at very high frequency concomitantly with bona fide transposon mutagenesis. This silent Mu insertion in Mu-sensitive strains, therefore, leads to a double-mutagenesis procedure and could be at the origin of some inconsistencies reported in genetic screens performed via RP4-mediated transposon mutagenesis using S17-1 λ*pir* or SM10 λ*pir* strains. To circumvent this problem, we constructed a new Mu-free *E. coli* donor strain, MFD*pir* (for Mu-free donor), that will simplify the analysis of future mutant hunts performed in *E. coli* and other Mu-sensitive RP4 host bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All experiments were carried out in lysogeny broth (LB) medium (MP Biomedicals, LLC; 10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) at 37°C (5). When required, antibiotics were added to the medium at the following concentrations: ampicillin (Ap) (100 μg/ml), apramycin (Apra) (30 μg/ml), chloramphenicol (Cm) (25 μg/ml), erythromycin (Erm) (200 μg/ml), kanamycin (Km) (50 μg/ml), streptomycin (Sm) (100 μg/ml), tetracycline (Tc) (7.5 μg/ml), and zeocin (Zeo) (50 μg/ml). Detection of the Lac^+ phenotype was done on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM). *dapA*::*pir116-erm* mutants were grown in medium supplemented with 0.3 mM diaminopimelic acid (DAP). Most gene deletions and replacements with an antibiotic resistance cassette were performed using the λ Red linear DNA gene replacement system and the 3-step PCR procedure described previously (16) and on our website (http://www.pasteur.fr/recherche/unites/Ggb/3SPCRprotocol.html). Briefly, the 500-bp left and 500-bp right regions flanking the gene to be deleted were amplified using gene.500–5/gene.cassette.L-3 and gene.cassette.L-5/gene.500–3 primer pairs, respectively (Table 2) and fused to the antibiotic resistance cassette by PCR. Alternatively, some constructions were made using linear DNA obtained in a single-step procedure; in this case, the PCR products of the DNA fragment to be inserted were flanked by 40-bp sequences homologous to the target region and amplified using primers gene.cassette.TL-5 and gene.cassette.TL-3 (Table 2). The PCR products were finally introduced by electroporation into cells expressing λ Red recombinase from plasmid pKOBEG or pKOBEGA, and transformants were recovered on selective LB agar plates. Mutations were confirmed by PCR amplification of the targeted genomic region using primers gene.ext-5 and gene-.ext-3 (16). The sequences of all the primers used are given in Table 2. Mutations were moved between strains by P1*vir* transduction.

TABLE 1. *E. coli* strains and plasmids used in this study

Name	Genotype or main characteristics	Antibiotic resistance	Reference
Strains			
JW2669	BW25113 Δ*recA*::*kan*	Km^r	2
MFD*pir*	MG1655 RP4-2-Tc::[ΔMu1::*aac(3)IV*-Δ*aphA*-Δ*nic*35-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA*	$Apra^r$ Zeo^r Erm^r	This study
MG1655	K-12 wild-type strain		6
MG1655-s	Spontaneous streptomycin-resistant mutant of MG1655		Laboratory collection
MG1655 Δ*lac*	MG1655 Δ*lacIZ*::*cat* Lac^-	Cm^r	This study
MG1655-s Δ*lac*	MG1655-s Δ*lacIZ*::*frt* Lac^-	Sm^r	This study
S17-1 λ*pir*	(F^-) RP4-2-Tc::Mu *aphA*::Tn*7recA*λ*pir*lysogen	Sm^r Tp^r	40
SM10 λ*pir*	(F^-) RP4-2-Tc::Mu *recA* λ*pir* lysogen	Km^r	40
ω7249	(F^-) RP4-2-Tc::Mu Δ*nic35* Δ*dapA*::(*erm-pir*)	Km^r Erm^r	3
Plasmids			
pKD3	Source of the *cat*::FRT cassette	Ap^r Cm^r	12
pKOBEG	*ori*R101*ts araC* arabinose-inducible λred γβα operon	Cm^r	9
pKOBEGA	pKOBEG derivative	Ap^r	9
pSC189	*oriT* Π-dependent *ori* R6K *mariner*-based transposon TnSC189	Km^r Ap^r	11
pSC189-Cm	Like pSC189 but carries TnSC189 Δ*kan*::*cat*	Cm^r Ap^r	F. Leroux
pCP20	Rep(Ts) Flp^+	Cm^r Ap^r	10

Bacterial mating and pSC189 mutagenesis. The recipient strain and the donor strain with or without the pSC189 plasmid were grown to an optical density at 600 nm (OD_{600}) of 0.6, at 37°C with aeration, washed twice, and resuspended in fresh LB medium to a similar OD_{600}. Six hundred microliters of the donor strain and 200 μl of the recipient strain were mixed. The bacteria were pelleted by centrifugation, resuspended in 30 μl of medium, and spotted on 0.45-μm nitrocellulose filters, which were placed on LB agar plates. After 24 h at 37°C, bacterial cells were recovered from the filter in 1.5 ml LB. Serial dilutions of the suspension were made and plated on selective media. When appropriate, 10 mM sodium citrate was added to the agar plates during mating and during transconjugant selection. Finally, several transconjugants were selected and screened for the Lac^+ phenotype (blue colonies on X-Gal), *lacIZ* insertion (by PCR, using primers LACZ.ACTIV.A1.500–5 and LacZatg+100–3), or Mu insertion (by PCR, using primers Mu1470bp-5 and Mu1470bp-3). The percentage of transconjugants containing pSC189, which could have been stabilized by transfer of λ*pir* to the recipient strain, was determined by plating on ampicillin-containing plates and stood below 2% in all matings performed.

Localization of bacteriophage Mu insertions in the chromosome. The site of the Mu insertion was determined by arbitrary primed PCR (31). One microliter of a suspension of the mutant to be analyzed was added to 50 μl of a PCR mixture (1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each deoxynucleoside triphosphate [dNTP], 20 mM Tris-HCl, pH 8.4, 0.05 U/μl LA *Taq* [TaKaRa]) containing 0.6 μM randomized primers ARB1 and ARB6 and 1.2 μM MuL.200–3, which hybridized the left-end region of Mu. The amplification conditions were as follows: 5 min at 94°C; six cycles of 30 s at 94°C, 30 s at 30°C, and 3 min at 72°C; 30 cycles of 30 s at 94°C, 30 s at 45°C, and 3 min at 72°C; and 5 min at 72°C. A second round of PCR was subsequently performed after dilution of 1 μl from the first PCR mixture into 50 μl of a fresh PCR mixture. This second amplification (40 cycles of 30 s at 94°C, 30 s at 60°C, and 3 min at 72°C; 5 min at 72°C) was carried out with 1.2 μM ARB2 and 1.2 μM MuL.100–3 as primers. The PCR products were purified by migration on agarose gels and sequenced using MuL.100–3. The sequence flanking the right-end region of Mu was determined similarly using MuR.200–5 and MuR.100–5, respectively.

Detection of Mu in S17-1 λ*pir* and SM10 λ*pir* culture supernatants. S17-1 λ*pir* and SM10 λ*pir* cells were grown at 37°C in LB until late exponential phase (OD_{600} = 1). The supernatant was harvested and filter sterilized (0.22 μm). Twenty microliters was spotted onto a lawn of Mu-sensitive bacteria inoculated to an OD_{600} of 0.001 in fresh LB agar (0.75% [wt/vol]) supplemented with 10 mM $MgSO_4$ and 20 mM $CaCl_2$ (42). The plates were incubated at 37°C overnight, and the emergence of plaques was examined the next day. In parallel, cultures of the sensitive strain in liquid LB containing 10 mM $MgSO_4$ and 20 mM $CaCl_2$ were inoculated to an OD_{600} of 0.05 and grown in the presence of 100 μl of serial dilutions of S17-1 λ*pir* or SM10 λ*pir* supernatants. The presence of Mu was assessed by premature lysis of the culture. Both kinds of experiments were carried out in LB supplemented with 50 mM sodium citrate as a negative control.

Construction of the new Mu-free donor strain MFD*pir*. The two copies, Mu1 and Mu2, flanking RP4 in ω7249 were deleted and replaced by cassettes encoding resistance to apramycin or zeocin, respectively. The cassettes were obtained by PCR amplification using the Phusion Flash High-Fidelity PCR Master Mix (Finnzymes). The primers MuL-apra-L3 and tetR.MuR.apra.L-5 were designed so that the apramycin cassette removed the Mu1 copy, with the exception of 640 bp at the 5′ end and the *′tetA-tetR* remaining downstream region; as for the zeocin resistance cassette, the primers tetA.zeo.L-3 and glvB.zeo.L-5 were designed to remove the entire Mu2 copy and the *tetA″* remaining upstream region (Table 2). The new construct was then transduced into MG1655, and the absence of any Mu was confirmed by PCR using Mu1470bp.500–5 and Mu1470bp.500–3. In order to remove the RP4-associated kanamycin resistance marker, most of the *aphA* gene of RP4 was replaced by the *cat*-Flp recombination target (FRT) cassette carried by pKD3 (12). This cassette was amplified by PCR using the primers apha.Cmfrt.TL-5 and apha.Cmfrt.TL-3. The chloramphenicol resistance marker was subsequently excised using the Flp recombinase-encoding plasmid pCP20, as previously described (10). In order to enable replication of R6K plasmids in this strain, the *dapA*::*pir116-erm* mutation, which codes for the Π protein, was introduced by P1*vir* transduction, and transductants were checked by PCR using primers dapA.100–5 and dapA.100–3 (15). Finally, possibilities for unwanted internal homologous recombination were reduced by introducing the Δ*recA*::*kan* mutation. To do so, the Δ*recA*::*kan* from the Keio collection was amplified by PCR using primers recA.500–5 and recA.500–3 and integrated into the chromosome by λ Red recombination (2). The associated kanamycin resistance marker was excised using pCP20 (2, 10). The inability of MFD*pir* to produce Mu particles in culture supernatant was confirmed as described above.

RESULTS

Evidence for bacteriophage Mu transfer between donor and recipient strains during transposon mutagenesis using *E. coli* S17-1 λ*pir* or SM10 λ*pir*. Bacterial genetic screens often involve RP4-mediated mobilization of *pir*-dependent suicide vectors that deliver transposons into the genomes of *pir*-less recipient strains, in which they cannot replicate. During the course of such a genetic analysis, we used the well-known donor strain *E. coli* S17-1 λ*pir*(pSC189) to deliver a TnSC189 kanamycin-resistant *mariner*-based transposon into *E. coli* MG1655 Δ*lac* (11). Intriguingly, we characterized a transconjugant with a transposon inserted into a region corresponding to bacteriophage Mu DNA. Considering the absence of Mu in the original recipient strain and the presence, by construction,

TABLE 2. Primers used in this study

Strain construction and analysis	Name	Sequence
Δ*lacIZ*::*cat*	lacI.ext-5	5′-GGGCGATCTGTTGCGCGAAG-3′
	lacI.500-5	5′-CTTATCCTTTCACCGGGCAATG-3′
	lacI.cat.L-3	5′-CTGCGAGTGATCTTCCGTCACAGGATTCACCACCCTGAATTGACTCT-3′
	lacZ.cat.L-5	5′-GATGAGTGGCAGGGCGGGGCGTAATAATAACCGGGCAGGCCATGTC-3′
	lacZ.500-3	5′-CGACCAAATTCGAAATTACTG-3′
	lacZ.ext-3	5′-CAAACTGATTATTGATGGTGAACA-3′
lacI-lacZ intergenic region	LACZACTIV.A1.500-5	5′-CTCACTCGCAATCAAATTCAG-3′
	lacZATG+100-3	5′-GGGGGATGTGCTGCAAGGCGATTAAG-3′
Random-primed PCR	ARB1	5′-GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT-3′
	ARB2	5′-GGCCACGCGTCGACTAGTAC-3′
	ARB6	5′-GGCCACGCGTCGACTAGTACNNNNNNNNNACGCC-3′
Localization of Mu	MuR200-5	5′-AATTTAATCAGTATCGCTAC-3′
	MuR100-5	5′-ATGTAATGAATAAAAAGCA-3′
	MuL200-3	5′-GTTTTGAACGTTTTTTGAAG-3′
	MuL100-3	5′-CTAAAATTTGCACTACAGGC-3′
Detection of Mu	Mu1470bp.500-5	5′-GTTACTTTTCAAAATTTAAAC-3′
	Mu1470bp.500-3	5′-CGCAGATAATCTGCAATCAG-3′
ΔMu1::*apra*	MuL.apra.ext-5	5′-ACATTGGATTATTCGGGATC-3′
	Mu.apra.500-5	5′-CGGGATCTGATGGGATTAGATTTG-3′
	MuL.apra.L-3	5′-CAAGGGCTCCAAGGATCGGGCCTTGATAAACAAATTAACCACAACC-3′
	tetR.MuR.apra.L-5	5′-CGCCAGTCGATTGGCTGAGCTCATGATCAGGACCGCTGCCGGAG-3′
	tetR.apra.500-3	5′-CGGCGTTTCTGGCGCGTTTG-3′
	tetR.apra.ext-3	5′-CCACATCATCTGTGGGAAACTC-3′
ΔMu2::*zeo*	tetA.zeo.ext-5	5′-ATATTTCGCTATTCTGGAGC-3′
	tetA.zeo.500-5	5′-GGTGTTGACACCAAACGCAG-3′
	tetA.zeo.L-3	5′-GTCAACACGTGCTCGGATCCAGAATCGTGGAAACGATAGGCCTATG-3′
	glvB.zeo.L-5	5′-TTCGTGGCCGAGGAGCAGGACTGAACAGCTCGATAGCTTAATTAATT C-3′
	glvB.500-3	5′-GCATGTCACAGATGTTGAGG-3′
	glvB.ext-3	5′-GCGGGATATCGCCGACCACC-3′
Δ*aphA*::*cat*-FRT	apha.Cmfrt.ext-5	5′-ACTACGAAATCGCCTACAGC-3′
	apha.Cmfrt.TL-5	5′-TGAAATGGTGAGATTGCGTTGGCTGGCGGGGCACATTTCTTAATGTGTA GGCTGGAGCTGCTTC-3′
	apha.Cmfrt.TL-3	5′-CCCGCCCAACGTCGATGCAGCCGACTACCTTACCTTCGATTAGTTCCTAT TCCGAAGTTC-3′
	apha.Cmfrt.ext-3	5′-GCATGAAAATGGCAAATAAC-3′
Δ*recA*::*kan*-FRT	recA.ext-5	5′-GTGCTATCTTGTCCGGCATA-3′
	recA.500-5	5′-GTTAAGTGAACAGGTTGGGC-3′
	recA.500-3	5′-CAGAAAACGCTGGATCTTAAC-3′
	recA.ext-3	5′-CTTCCATCAGATAGCCACGA-3′
Δ*dapA*::*pir116-erm*	dapA.100-5	5′-TGAGTTGTTCTTAAGGAAAG-3′
	dapA.100-3	5′-CAGGCAGCGAGTAATAAAACAAG-3′

of an integrated Mu derivative in *E. coli* S17-1λ*pir* (RP4-2-Tc::Mu) (see the introduction) (Fig. 1), we investigated the potential silent transfer of Mu from *E. coli* S17-1 λ*pir* into recipient cells. We screened more than 100 transconjugants by PCR using primers specific for Mu transposase gene *A*, and we indeed found that, in addition to TnSC189 transposon insertion, 63% ± 13% of them also carried bacteriophage Mu DNA into their chromosomes (Table 3). Moreover, a high frequency of Mu (61% ± 17%) was also observed in recipient cells when conjugation was performed using a plasmid-free S17-1 λ*pir*, suggesting that the transfer of Mu is independent of the bona fide transposon delivery (Table 3). The use of the alternative strain SM10 λ*pir* also led to frequent transfer of Mu into *E. coli* recipients, thereby demonstrating that the popular donor strains *E. coli* S17-1 λ*pir* and SM10 λ*pir* are sources of very significant contamination by bacteriophage Mu (Table 3).

Release of Mu phage particles in *E. coli* S17-1 λ*pir* and SM10 λ*pir* culture supernatants. The presence of an RP4-2-Tc::Mu insertion into the genome of *E. coli* S17-1 λ*pir* or SM10 λ*pir* qualifies these two strains as Mu lysogens that should spontaneously release Mu at a frequency of 10^{-4} bacteria (47). In order to check for the presence of free Mu phage particles in *E. coli* S17-1 λ*pir* and SM10 λpir culture supernatants, we spotted a drop of filter-sterilized supernatant onto a

TABLE 3. Frequencies of Mu and Lac$^+$ transfers during conjugation with SM10 λ*pir* and S17-1 λ*pir* donor strain derivatives

Donor [relevant characteristics] (plasmid)[a]	Recipient	Transconjugant frequency[b]	% Mu transfer[c]	% Lac$^+$[c]
S17-1 λ*pir* [ChRP4 *pir*$^+$] (pSC189)	MG1655 Δ*lac*	$1.7 \times 10^{-2} \pm 5.5 \times 10^{-3}$	63 ± 13	0.014 ± 0.009
S17-1 λ*pir* [ChRP4 *pir*$^+$] (pSC189) with 10 mM sodium citrate	MG1655 Δ*lac* with 10 mM sodium citrate	$3.4 \times 10^{-3} \pm 1.8 \times 10^{-3}$	35 ± 34	ND[d]
S17-1 λ*pir* [ChRP4 *pir*$^+$]	MG1655 Δ*lac*	ND	61 ± 17[e]	0.55 ± 0.37[e]
SM10 λ*pir* [ChRP4 *pir*$^+$] (pSC189-Cm)	MG1655-s Δ*lac*	$6.3 \times 10^{-2} \pm 5.4 \times 10^{-2}$	89 ± 10	<0.001
ω7249 [ChRP4Δ*nicpir*$^+$] (pSC189-Cm)	MG1655-s Δ*lac*	$1.2 \times 10^{-3} \pm 3.2 \times 10^{-4}$	93 ± 3	<0.001
MFD*pir* [ChRP4 Δ*nic* ΔMu *pir*$^+$] (pSC189-Cm)	MG1655-s Δ*lac*	$2.0 \times 10^{-3} \pm 1.0 \times 10^{-3}$	<0.72	<0.001

[a] ChRP4, chromosomal integration of RP4-2-Tc::Mu.
[b] Expressed as the mean number ± standard deviation of transconjugants per recipient cell.
[c] Expressed as the percentage ± standard deviation of events in the transconjugant population. The percentage of Mu transfer was determined by PCR on 46 colonies per mating. The percentage of Lac$^+$ colonies was determined by plating on X-Gal-containing agar plates. Three independent matings were done for each condition.
[d] ND, not determined.
[e] Expressed as the percentage ± standard deviation of events in the exconjugant recipient population.

lawn of Mu-sensitive MG1655 Δ*lac* or Mu-resistant MG1655 Δ*lac* already carrying a Mu bacteriophage. Incubation at 37°C revealed confluent lysis plaques in the case of the Mu-sensitive MG1655 Δ*lac* strain only, confirming that the lysis was Mu dependent and that Mu particles were released by the donor strains into the medium (Fig. 2 and data not shown). Consistently, the addition of filter-sterilized S17-1 λ*pir* supernatant into an exponential-phase liquid culture of MG1655 Δ*lac* led to cell lysis, as monitored by a decrease in culture turbidity (data not shown). PCR analysis of the surviving bacteria after they were plated on LB agar plates showed that 22% of them had acquired a Mu prophage.

Mapping RP4-2-Tc::Mu insertions in *E. coli* S17-1 λ*pir* and SM10 λ*pir*. *E. coli* S17-1 λ*pir* and SM10 λ*pir* were constructed in the early 1980s, but the location and mechanism of RP4-2-Tc::Mu insertion into the *E. coli* chromosome have been poorly described (40). We therefore mapped the location of Mu insertion in *E. coli* S17-1 λ*pir* by random-primed PCR using primers specific for the left and right extremities of the Mu sequence, and we determined that two potentially functional copies of Mu flanked the integrated RP4 replicon inserted in *mhpC* at position 371203 of the *E. coli* chromosome, close to the *lacZ* gene (position 365529) (Fig. 3A). The two copies of Mu are in the same orientation, indicating that integration of RP4-2-Tc::Mu into the original *E. coli* 294 background arises from a Mu-driven replicon fusion mechanism (47). As the integration of RP4-2-Tc::Mu occurred independently in the two donor strains (see the introduction), we also determined that the two copies of Mu flanking the RP4 region in SM10 λ*pir* were at position 3859682 on the chromosome in the *glvB* gene (minute 83). This location is consistent with genetic evidence provided in 1983 by Simon et al., who mapped the RP4-2-Tc::Mu integration site in SM10 λ*pir* next to the methionine marker in the *met ilv arg* region (minute 89) (40). We also determined that an additional copy of the Mu prophage was located outside the RP4-2-Tc::Mu region in SM10 λ*pir*, at position 2450790 (minute 53) in the *yfcU* gene.

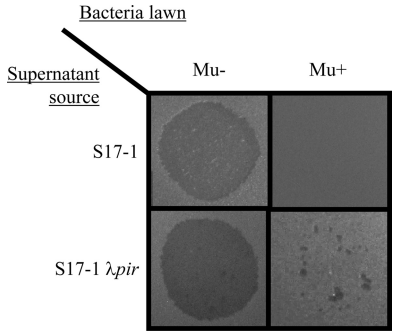

FIG. 2. Mu particles are released into the supernatant of S17-1 and S17-1 λ*pir* cultures. Confluent lysis was observed when a drop of filter-sterilized supernatant of S17-1 or S17-1 λ*pir* culture was spotted onto a lawn of growing MG1655 Δ*lac* cells (Mu$^-$). In contrast, no (S17-1 supernatant) or very few (S17-1 λ*pir* supernatant) plaques appeared when the same supernatants were spotted on a lawn of a Mu lysogenic derivative (Mu$^+$), confirming that the lysis is Mu dependent and that Mu particles are present in the supernatant. Mu-independent lysis observed using S17-1 λ*pir* supernatant probably results from release of λ phage particles into the medium.

Evidence for *hfr* Mu transfer into recipient cells. We took advantage of the insertion of RP4-2-Tc::Mu in the vicinity of *lacZ* on the chromosome of S17-1 λ*pir* to evaluate the possibility of transfer of RP4-2-Tc::Mu flanking regions into MG1655 Δ*lac* (a Lac$^-$ strain) by *hfr* transfer (35, 40, 50). As shown in Table 3, a large proportion of MG1655 Δ*lac* transconjugants obtained with S17-1 λ*pir* or S17-1 λ*pir*(pSC189) as donor strains and plated on X-Gal-containing agar plates displayed a Lac$^+$ phenotype (blue colonies). Using primers hybridizing on the *lacIZ* region initially absent from MG1655 Δ*lac*, we amplified a product corresponding to the wild-type *lacIZ* region in all tested blue transconjugants, indicating that this Lac$^+$ phenotype resulted from the insertion of a functional *lacIZ* region (data not shown). Moreover, the use of primers hybridizing on the left extremity of Mu showed that this functional *lacIZ* region was genetically linked to Mu (Fig. 3B). Finally, conjugations performed in MG1655-s Δ*lac* using a donor strain carrying a mutation in an RP4 *oriT nic* site that impairs transfer of chromosomal DNA, ω7249(pSC189-Cm), did not produce any transconjugants with a Lac$^+$ phenotype (3). These results confirmed that *hfr*-mediated cotransfer of Mu and the adjacent *lac* region is a frequent event when S17-1 λ*pir* is used. In contrast, transconjugants displaying a Lac$^+$ phenotype were never observed when *E. coli* SM10 λ*pir* was used as the donor strain, which is consistent with the RP4-2-Tc::Mu insertion position in the strain (minute 83), distant from the *lacZ* gene (minute 8). Interestingly, conjuga-

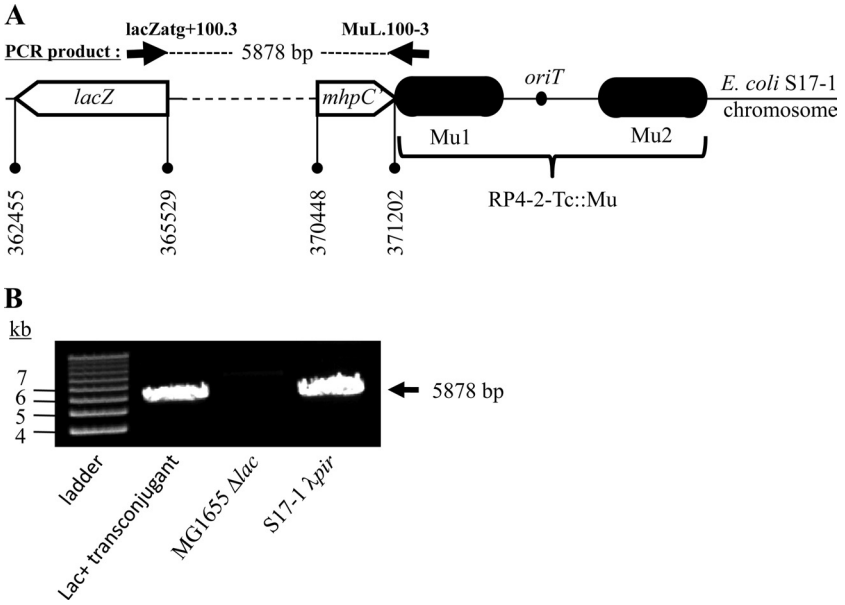

FIG. 3. Genetic map of Mu insertions in *E. coli* S17-1λ*pir*. (A) Structure of the RP4-2-Tc::Mu insertion region in *E. coli* S17-1 λ*pir* showing the *lacZ* region and the *oriT*-carrying RP4 core plasmid flanked by the two copies of bacteriophage Mu, Mu1 and Mu2, in the same orientation. (B) PCR analysis of the junction between Mu and *lacZ* in S17-1 λ*pir*, MG1655 Δ*lac*, and a random Lac^+ transconjugant obtained by conjugation between these strains, using primers lacZatg+100–3 and MuL.100–3. The region amplified using these primers is shown in panel A.

tion using the *hfr*-defective strain ω7249(pSC189-Cm) still produced a high frequency of Mu-carrying transconjugants (93% ± 3%). These data therefore suggested that *de novo* infection via Mu phage particles released by the donor strains S17-1 λ*pir* and SM10 λ*pir* may occur concomitantly with conjugative transfer of transposon delivery plasmids and not exclusively by *hfr* transfer (Fig. 4).

Site-directed removal of Mu from RP4-2-Tc::Mu-containing donor strains leads to Mu-free delivery of transposons to recipient strains. The presence of Mu prophages in donor strains results in undesirable markerless insertion events in recipient strains that interfere with analysis of the mutagenesis outcome. To try to inhibit Mu infection of recipient bacteria, we performed mating in the presence of 10 mM sodium citrate, but we still observed high frequencies of Mu transfers in this case (around 30% of the transconjugants) (Table 3). Therefore, to circumvent the problem, we decided to construct a new Mu-free strain. We chose the SM10 derivative ω7249 as a template to take advantage of the Δ*nic35* mutation introduced into the RP4-2-Tc::Mu *oriT* locus, which prevents the transfer of chromosomal DNA by *hfr* (3). The analysis of the RP4 region of SM10 λ*pir* revealed that integration of RP4-2-Tc::Mu into the

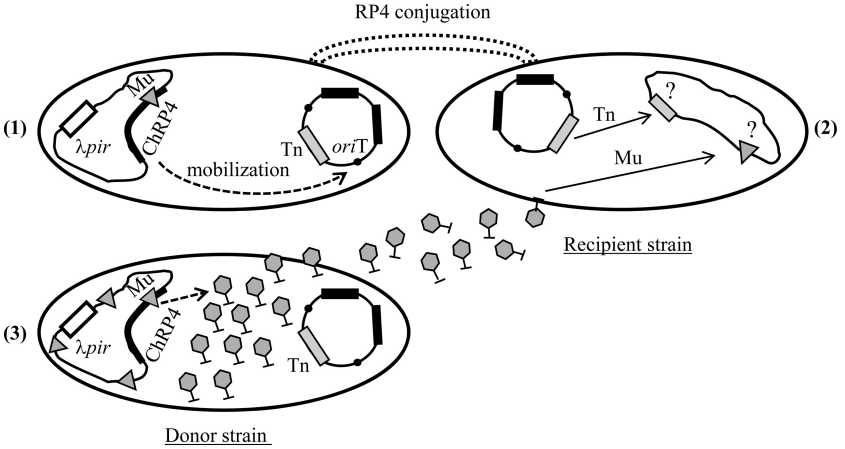

FIG. 4. *De novo* infection by Mu during conjugation results in a second mutation event in the recipient strain. (Step 1) During conjugation, pSC189 is transferred to the recipient cell using the mobilization and conjugation functions of the RP4 plasmid integrated into the chromosomes of donor strains S17-1 λ*pir* and SM10 λ*pir* (ChRP4). (Step 2) Once in the cell, the pSC189-carried transposon (gray box) integrates into the chromosome, altering the expression of a gene and its associated function. (Step 3) In parallel, the ChRP4-associated Mu prophage (triangle) is induced spontaneously into a subpopulation of donor cells. Mu particles (tailed hexagons) are then released into the environment and infect the recipient cells, leading, in some cases, to a second mutagenic event by lysogenization (2).

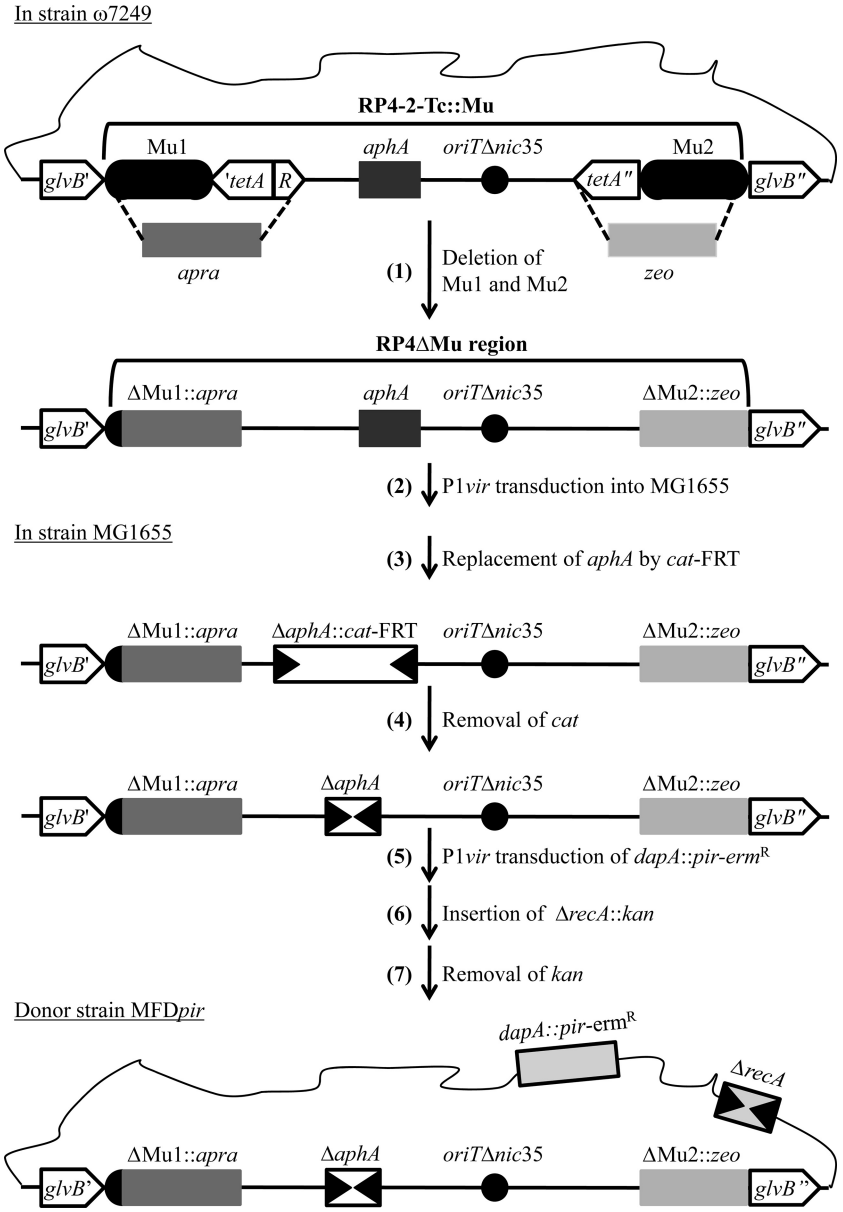

FIG. 5. Construction of a new donor strain, MFD*pir* (see the text for details). The introduction of RP4-2-Tc::Mu into the chromosome of SM10 and that of its derivative, ω7249, occurred in *glvB*. This resulted in duplication of Mu, with each copy (Mu1 and Mu2) flanking the core RP4 and displaying the same orientation. The two Mu1 and Mu2 copies, along with the remains of the interrupted tetracycline resistance region (*'tetA-R* and *tetA"*), were removed and replaced by cassettes providing resistance to apramycin (*apra*) and zeocin (*zeo*), respectively. After transduction of the Mu-free RP4 region into MG1655, the kanamycin resistance gene *aphA* was replaced by a *cat*-FRT cassette; the chloramphenicol resistance gene, *cat*, was subsequently excised using Flp recombinase. Then, the Π-encoding *dapA*::*pir116-erm* locus was introduced into the strain. The *recA* gene was finally replaced by a *kan*-FRT cassette, and the kanamycin resistance gene, *kan*, was removed with Flp, giving the new donor strain, MFD*pir*.

chromosome led to the duplication of Mu, with each copy (termed Mu1 and Mu2, respectively) flanking the RP4 core plasmid (Fig. 5). In order to remove Mu from this strain, we replaced Mu1 and Mu2 with genetic elements conferring apramycin and zeocin resistance, respectively (Fig. 5, step 1). This procedure also enabled us to delete the remains of the *tetRA* region that had originally been interrupted by Mu in RP4-2-Tc::Mu (Fig. 5, step 1). We then transduced the RP4 ΔMu region from ω7249 ΔMu1::*apra* ΔMu2::*zeo* into MG1655, and we verified that the resulting strain did not contain any other copy of Mu (Fig. 5, step 2). To reduce the number of antibiotic resistance markers and to make the strain compatible with most available delivery vectors, the kanamycin resistance gene carried by the RP4ΔMu region was deleted and replaced by an excisable *cat*-FRT chloramphenicol resistance cassette that was subsequently removed using Flp recombinase (Fig. 5, steps 3 and 4) (10). We then introduced the *pir* locus *dapA*::*pir-erm* from ω7249 and a Δ*recA*::*kan*-FRT mutation to

reduce the efficiency of an internal homologous recombination (Fig. 5, steps 5 and 6). Finally, the kanamycin resistance marker was removed using Flp recombinase to produce the new Mu-free donor strain, MFD*pir* (Fig. 5, step 7).

In order to demonstrate that the RP4 region of MFD*pir* was still functional, we introduced the Π-dependent pSC189-Cm plasmid and performed conjugative mating with the recipient strain, MG1655-s Δ*lac*. The frequency of the transconjugants carrying the TnSC189-Cm transposon (Cm^r) was similar to that observed using the parental strain, ω7249, suggesting that the alterations we made in the RP4 region did not affect the conjugation process (Table 3). While transconjugant frequency using MFD*pir* is slightly lower than that observed with SM10 λ*pir* or S17-1 λ*pir*, it is similar to the transconjugant frequency obtained with the parental Δ*nic* strain, ω7249 (3).

The transconjugants tested were all Lac^- and devoid of any Mu sequence, as shown by PCR, confirming the absence of Mu or *lac* transfer when the new Mu-free donor strain was used (Table 3).

Altogether, these results indicate that the new MFD*pir* donor strain is an efficient alternative to the use of S17-1 λ*pir* and SM10 λ*pir* donors and can be used to introduce exogenous DNA into a target strain, thus avoiding undesirable side effects resulting from the presence of Mu in currently available donors derived from the original S17-1 λ*pir* and SM10 λ*pir* strains.

DISCUSSION

In spite of—or because of—the wealth of information provided by genome sequencing and subsequent microarray gene expression analyses, the functions of a large fraction of identified bacterial genes remain unknown or have not been experimentally validated, even in well-studied model organisms such as *E. coli* (22). Most gene function studies rely on direct investigation of bacterial-gene function through creation of mutants. While different PCR-based site-directed mutagenesis strategies were developed in the past decade to facilitate such analysis, random genetic screens aimed at identifying the genetic basis of phenotypes expressed under different growth conditions or environments remain a very fruitful approach (4, 23, 26, 32, 38, 39). In many bacteria, random mutagenesis is performed via the introduction of transposons carrying antibiotic resistance markers into the chromosome of the target strain (20). The introduction of the inactivating element by conjugation of a suicide vector therefore remains the method of choice for performing cheap, broad, and relatively unbiased genomic screens.

The integration of RP4 mobilization functions in *E. coli* strains S17-1 and SM10 by Pülher and collaborators was originally designed to allow the conjugative transfer of foreign genes in non-*E. coli* Gram-negative bacteria included in the RP4 host range, such as *Rhizobiaceae* (40). Although state of the art at the time, it also allowed the incidental introduction of a functional Mu prophage into these strains and their respective λ*pir* derivatives, which have since been widely used for conjugation transfer of exogenous DNA, including suicide transposon-carrying vectors, to a large number of bacterial species (*E. coli* [4, 23, 29], *Klebsiella pneumoniae* [7], *Edwardsiella ictaluri* [46], *Rhizobium meliloti* [33], *Vibrio cholerae* [28], and *Erwinia carotovora* [32]). In some cases, it was reported that transconjugant transposon mutants obtained using S17-1 and SM10 donor strains did not display the expected genetic linkage between the screened phenotype and insertion events or phenotype instability in transconjugants, but the reasons for these inconsistencies were not investigated (26, 38). In this study, we show that both *E. coli* S17-1 λ*pir* and SM10 λ*pir* donor strains transfer Mu bacteriophage at high frequencies into recipient *E. coli* cells by both *hfr* and *de novo* infection. Our data demonstrate in particular that Mu transfer to the recipient cell can be independent of the conjugative process and mainly results from infection by Mu phage particles released into the culture medium by *E. coli* S17-1 λ*pir* and SM10 λ*pir* donor strains. Since we observed Mu transfer using S17-1 λ*pir*, SM10 λ*pir*, and ω7249 coming from many independent sources (different laboratory collections), this suggests that Mu contamination is not specific to our own strain collection but rather is an intrinsic feature of the original S17-1 λ*pir* and SM10 λ*pir*. Moreover, in addition to the conditions used to perform conjugation, we randomly selected two other mating procedures in the literature (38, 40). Despite strong differences between these procedures regarding mating duration (2 h or 24 h), media (LB or RMGC), and growth conditions (on plates or in broth), we observed a high frequency of Mu transfer to the transconjugants in each case (data not shown), suggesting that S17-1 λ*pir*- and SM10 λ*pir*-mediated Mu contamination occurs efficiently when classical conjugation protocols are used. Therefore, we speculate that many inconsistencies observed during S17-1 λ*pir*- and SM10 λ*pir*-based screens performed on Mu-sensitive bacteria could result from infectious and silent transfer of Mu from donor to recipient strains, in addition to the expected transposon insertion. This second mutagenic event is indeed likely to affect the outcome of many site-directed mutagenesis experiments and to complicate correct gene function assignment. Moreover, Mu is known to replicate by transposition, inducing subsequent DNA rearrangements and therefore contributing to genetic instability and highly variable phenotypes (47). However, despite these shortcomings and to the best of our knowledge, infection of other recipient strains by bacteriophage Mu in transposon mutagenesis experiments has not been reported. This may be due to the fact that not all potential recipient strains display equal sensitivity to Mu infection. Mu phage particles indeed infect their target cells by binding to terminal Glcα1-2Glcα1 or GlcNAcα1-2Glcα1 oligosaccharide residues in the lipopolysaccharide (LPS) outer end (36). As a consequence, Mu-resistant strains are expected to be less affected or unaffected by the double-mutagenesis event occurring during conjugation. Indeed, we did not observe any transfer of Mu from the S17-1 λ*pir* donor to uropathogenic *E. coli* strain CFT073, which carries the LPS core type R1 and is not recognized by Mu, when performing mating between these two strains (data not shown). The natural Mu host range is rather limited but includes many pathogenic bacteria, such as *Salmonella enterica* subsp. *arizonae*, *Shigella dysenteriae* (43), *Citrobacter freundii* (13), and *Erwinia chrysanthemi* (49), and clinical strains of *E. coli* that harbor the Mu-sensitive LPS core type R2 (1, 18, 36). While transfer of Mu during mutagenic conjugation may reveal some phenotypes that can only be observed with two independent mutations, it remains an important barrier to the proper genetic characterization of these organisms, some of which are

being used as model bacteria. Furthermore, while Mu insertions are restricted to Mu-sensitive bacteria, this still represents a significant number of strains, considering all natural isolates of each species now studied to explore bacterial biodiversity (34, 44, 48). Finally, Mu-resistant recipient strains are not absolutely protected from Mu contamination, since Mu can also enter the cell via *hfr*. This may seriously impair the outcome of mutagenesis performed on resistant strains, since Mu has been reported to develop in many bacteria besides *E. coli* (*Enterobacteriaceae*, *Rhizobiaceae*, *Pseudomonas*, and other Gram-negative species), where it can generate progeny and chromosomal rearrangements (51).

Whereas alternative methods have been developed based on *in vivo* triparental mating or *in vitro* insertion of transposition complexes into the target strain by electroporation (19, 21, 41), these methods are costly or require appropriate combinations of strain and plasmid markers to allow selection of proper insertion events. To circumvent the problems caused by secondary Mu insertion, we constructed a novel donor strain, MFD*pir*, which combines both the improvements made recently by Babic and coworkers in preventing transfer of chromosomal DNA by an RP4-mediated *hfr* mechanism and the absence of any Mu prophage that could contaminate the recipient cells (3). This new donor strain remains as active as the previous S17-1 λ*pir* and SM10 λ*pir* strains in promoting mutagenic conjugation and leaves open the possibility of using transposons carrying many different resistance markers. In addition, its inability to grow in the absence of DAP facilitates counterselection of the donor strain after conjugation. The use of this new MFD*pir* strain should enable more straightforward analyses of transconjugants produced by future transposon mutagenesis in *E. coli* and other RP4 host bacteria.

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REFERENCES

1. **Appelmelk, B. J., Y. Q. An, T. A. Hekker, L. G. Thijs, D. M. MacLaren, and J. de Graaf.** 1994. Frequencies of lipopolysaccharide core types in *Escherichia coli* strains from bacteraemic patients. Microbiology **140:**1119–1124.
2. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**2006.0008.
3. **Babic, A., A. M. Guerout, and D. Mazel.** 2008. Construction of an improved RP4 (RK2)-based conjugative system. Res. Microbiol. **159:**545–549.
4. **Baca-DeLancey, R. R., M. M. South, X. Ding, and P. N. Rather.** 1999. *Escherichia coli* genes regulated by cell-to-cell signaling. Proc. Natl. Acad. Sci. U. S. A. **96:**4610–4614.
5. **Bertani, G.** 2004. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J. Bacteriol. **186:**595–600.
6. **Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1462.
7. **Boddicker, J. D., R. A. Anderson, J. Jagnow, and S. Clegg.** 2006. Signature-tagged mutagenesis of *Klebsiella pneumoniae* to identify genes that influence biofilm formation on extracellular matrix material. Infect. Immun. **74:**4590–4597.
8. **Bremer, E., T. J. Silhavy, and G. M. Weinstock.** 1985. Transposable lambda p*lacMu* bacteriophages for creating *lacZ* operon fusions and kanamycin resistance insertions in *Escherichia coli*. J. Bacteriol. **162:**1092–1099.
9. **Chaveroche, M. K., J. M. Ghigo, and C. d'Enfert.** 2000. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res. **28:**E97.
10. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli:* TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.
11. **Chiang, S. L., and E. J. Rubin.** 2002. Construction of a *mariner*-based transposon for epitope-tagging and genomic targeting. Gene **296:**179–185.
12. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
13. **de Graaff, J., P. C. Kreuning, and P. van de Putte.** 1973. Host controlled restriction and modification of bacteriophage Mu and Mu-promoted chromosome mobilization in *Citrobacter freundii*. Mol. Gen. Genet. **123:**283–288.
14. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
15. **Demarre, G., A. M. Guerout, C. Matsumoto-Mashimo, D. A. Rowe-Magnus, P. Marliere, and D. Mazel.** 2005. A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPα) conjugative machineries and their cognate *Escherichia coli* host strains. Res. Microbiol. **156:**245–255.
16. **Derbise, A., B. Lesic, D. Dacheux, J. M. Ghigo, and E. Carniel.** 2003. A rapid and simple method for inactivating chromosomal genes in *Yersinia*. FEMS Immunol. Med. Microbiol. **38:**113–116.
17. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U. S. A. **76:**1648–1652.
18. **Gibb, A. P., G. R. Barclay, I. R. Poxton, and F. di Padova.** 1992. Frequencies of lipopolysaccharide core types among clinical isolates of *Escherichia coli* defined with monoclonal antibodies. J. Infect. Dis. **166:**1051–1057.
19. **Goryshin, I. Y., J. Jendrisak, L. M. Hoffman, R. Meis, and W. S. Reznikoff.** 2000. Insertional transposon mutagenesis by electroporation of released Tn*5* transposition complexes. Nat. Biotechnol. **18:**97–100.
20. **Hayes, F.** 2003. Transposon-based strategies for microbial functional genomics and proteomics. Annu. Rev. Genet. **37:**3–29.
21. **Hoffman, L. M., J. J. Jendrisak, R. J. Meis, I. Y. Goryshin, and S. W. Reznikof.** 2000. Transposome insertional mutagenesis and direct sequencing of microbial genomes. Genetica **108:**19–24.
22. **Hu, P., S. C. Janga, M. Babu, J. J. Diaz-Mejia, G. Butland, W. Yang, O. Pogoutse, X. Guo, S. Phanse, P. Wong, S. Chandran, C. Christopoulos, A. Nazarians-Armavil, N. K. Nasseri, G. Musso, M. Ali, N. Nazemof, V. Eroukova, A. Golshani, A. Paccanaro, J. F. Greenblatt, G. Moreno-Hagelsieb, and A. Emili.** 2009. Global functional atlas of *Escherichia coli* encompassing previously uncharacterized proteins. PLoS Biol. **7:**e96.
23. **Hunstad, D. A., S. S. Justice, C. S. Hung, S. R. Lauer, and S. J. Hultgren.** 2005. Suppression of bladder epithelial cytokine responses by uropathogenic *Escherichia coli*. Infect. Immun. **73:**3999–4006.
24. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
25. **Lawley, T. D., K. Chan, L. J. Thompson, C. C. Kim, G. R. Govoni, and D. M. Monack.** 2006. Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. PLoS Pathog. **2:**e11.
26. **Létoffé, S., P. Delepelaire, and C. Wandersman.** 2006. The housekeeping dipeptide permease is the *Escherichia coli* heme transporter and functions with two optional peptide binding proteins. Proc. Natl. Acad. Sci. U. S. A. **103:**12891–12896.
27. **Merighi, M., A. Carroll-Portillo, A. N. Septer, A. Bhatiya, and J. S. Gunn.** 2006. Role of *Salmonella enterica* serovar Typhimurium two-component system PreA/PreB in modulating PmrA-regulated gene transcription. J. Bacteriol. **188:**141–149.
28. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
29. **Moxley, R. A., E. M. Berberov, D. H. Francis, J. Xing, M. Moayeri, R. A. Welch, D. R. Baker, and R. G. Barletta.** 1998. Pathogenicity of an enterotoxigenic *Escherichia coli* hemolysin (*hlyA*) mutant in gnotobiotic piglets. Infect. Immun. **66:**5031–5035.
30. **Murphy, K. C.** 1998. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. J. Bacteriol. **180:**2063–2071.
31. **O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter.** 1999. Genetic approaches to study of biofilms. Methods Enzymol. **310:**91–109.
32. **Pemberton, C. L., N. A. Whitehead, M. Sebaihia, K. S. Bell, L. J. Hyman,**

S. J. Harris, A. J. Matlin, N. D. Robson, P. R. Birch, J. P. Carr, I. K. Toth, and G. P. Salmond. 2005. Novel quorum-sensing-controlled genes in *Erwinia carotovora* subsp. *carotovora*: identification of a fungal elicitor homologue in a soft-rotting bacterium. Mol. Plant Microbe Interact. **18:**343–353.

33. **Pobigaylo, N., D. Wetter, S. Szymczak, U. Schiller, S. Kurtz, F. Meyer, T. W. Nattkemper, and A. Becker.** 2006. Construction of a large signature-tagged mini-Tn*5* transposon library and its application to mutagenesis of *Sinorhizobium meliloti*. Appl. Environ. Microbiol. **72:**4329–4337.
34. **Rasko, D. A., M. J. Rosovitz, G. S. Myers, E. F. Mongodin, W. F. Fricke, P. Gajer, J. Crabtree, M. Sebaihia, N. R. Thomson, R. Chaudhuri, I. R. Henderson, V. Sperandio, and J. Ravel.** 2008. The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. J. Bacteriol. **190:**6881–6893.
35. **Rosenberg, C., B. Bergeron, J. S. Julliot, and J. Denarie.** 1977. Use of RP4 plasmids carrying bacteriophage Mu insertions in nitrogen fixing bacteria *Klebsiella pneumoniae* and *Rhizobium meliloti*. Basic Life Sci. **9:**411–416.
36. **Sandulache, R., P. Prehm, and D. Kamp.** 1984. Cell wall receptor for bacteriophage Mu G(+). J. Bacteriol. **160:**299–303.
37. **Schweizer, H.** 2008. Bacterial genetics: past achievements, present state of the field, and future challenges. Biotechniques **44:**633–634, 636–641.
38. **Serina, S., F. Nozza, G. Nicastro, F. Faggioni, H. Mottl, G. Deho, and A. Polissi.** 2004. Scanning the *Escherichia coli* chromosome by random transposon mutagenesis and multiple phenotypic screening. Res. Microbiol. **155:** 692–701.
39. **Shuman, H. A., and T. J. Silhavy.** 2003. The art and design of genetic screens: *Escherichia coli*. Nat. Rev. Genet. **4:**419–431.
40. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology **1:**784–791.
41. **Stabb, E. V., and E. G. Ruby.** 2002. RP4-based plasmids for conjugation between *Escherichia coli* and members of the *Vibrionaceae*. Methods Enzymol. **358:**413–426.
42. **Tabor, H., E. W. Hafner, and C. W. Tabor.** 1983. Localized mutagenesis of any specific region of the *Escherichia coli* chromosome with bacteriophage Mu. Methods Enzymol. **94:**91–104.
43. **Taylor, A. L.** 1963. Bacteriophage-induced mutation in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **50:**1043–1051.
44. **Tenaillon, O., D. Skurnik, B. Picard, and E. Denamur.** 2010. The population genetics of commensal *Escherichia coli*. Nat. Rev. Microbiol. **8:**207–217.
45. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41:**77–101.
46. **Thune, R. L., D. H. Fernandez, J. L. Benoit, M. Kelly-Smith, M. L. Rogge, N. J. Booth, C. A. Landry, and R. A. Bologna.** 2007. Signature-tagged mutagenesis of *Edwardsiella ictaluri* identifies virulence-related genes, including a *Salmonella* pathogenicity island 2 class of type III secretion systems. Appl. Environ. Microbiol. **73:**7934–7946.
47. **Toussaint, A., and A. Résibois.** 1983. Phage Mu: transposition as a life-style, p. 105–158. *In* J. A. Shapiro (ed.), Mobile genetic elements. Academic Press, New York, NY.
48. **Valle, J., S. Da Re, S. Schmid, D. Skurnik, R. D'Ari, and J. M. Ghigo.** 2008. The amino acid valine is secreted in continuous-flow bacterial biofilms. J. Bacteriol. **190:**264–274.
49. **van de Putte, P., S. Cramer, and M. Giphart-Gassler.** 1980. Invertible DNA determines host specificity of bacteriophage Mu. Nature **286:**218–222.
50. **Van Gijsegem, F., and A. Toussaint.** 1982. Chromosome transfer and R-prime formation by an RP4::mini-Mu derivative in *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Plasmid **7:**30–44.
51. **Van Gijsegem, F., A. Toussaint, and M. Casadaban.** 1987. Mu as a genetic tool, p. 215–250. *In* N. Symonds, A. Toussaint, P. Van de Putte, and M. M. Howe (ed.), Phage Mu. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
52. **Zhang, X. X., and P. B. Rainey.** 2008. Dual involvement of CbrAB and NtrBC in the regulation of histidine utilization in *Pseudomonas fluorescens* SBW25. Genetics **178:**185–195.